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The human *ETS* gene family comprises 27 individual members and encodes sequence specific DNA binding proteins that regulate transcription. *PEA3* is the founding member of a subfamily of *ETS* genes, which also include *ER81* and *ERM*. *PEA3* is overexpressed in 76% of all human breast tumors and in 93% of the HER2-positive subclass of such tumors. PEA3 is also overexpressed in most human breast tumor cell lines by comparison to non-transformed immortalized human mammary epithelial cells. Furthermore, PEA3 transcripts are elevated in the mammary tumors of three different breast cancer prone transgenic mouse strains and inhibition of the function of PEA3 (and ER81 and ERM) in mammary epithelial cells abrogates mammary oncogenesis induced by Her2. These findings imply that PEA3 and its subfamily members are required for mammary oncogenesis by regulating the expression of genes whose products play cardinal roles in breast cancer. Our objective is to identify PEA3 regulated genes in human mammary tumor cells; such genes may encode diagnostic and prognostic markers and molecular targets for drug discovery. To this end we constructed and characterized recombinant human adenoviruses encoding PEA3 or dominant-negative PEA3 and assessed their capacity to infect and regulated expression of cellular genes in untransformed and transformed human mammary epithelial cells.

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INTRODUCTION

PEA3 is overexpressed in 76% of all human breast tumors and in 93% of the HER2-positive subclass of such tumors (Benz, O'Hagan et al. 1997). PEA3 is also highly expressed in most human breast tumor cell lines (de Launoit, Chotteau-Lelievre et al. 2000). Furthermore, PEA3 transcripts are elevated in the mammary tumors arising in each of three different breast cancer-prone transgenic mouse strains that express either polyomavirus middle tumor antigen, Her2 or Wnt-1 under the control of the mouse mammary tumor virus (MMTV) promoter (Trimble, Xin et al. 1993; Howe, Crawford et al. 2001). Analyses of global *ets* gene expression in Her2-induced mouse mammary tumors revealed the coordinate overexpression of all three *pea3* subfamily genes in mammary epithelial tumor cells (Shepherd, Kockeritz et al. 2001). Other *ets* genes expressed in the mouse mammary gland are not overexpressed in mammary tumors. Importantly inhibition of the function of the PEA3 subfamily proteins by a dominant-negative form of PEA3 (DNPEA3EN) retards mammary oncogenesis induced by Her2 in transgenic mice (Shepherd, Kockeritz et al. 2001). These latter findings imply a required role for PEA3 subfamily proteins or other DNA binding proteins with similar DNA binding specificity in mammary tumorigenesis. Because the PEA3 subfamily proteins regulate transcription and because this function is required for mammary oncogenesis these finding suggest that PEA3 subfamily regulated genes play important roles in mammary tumorigenesis. Our objective is to identify PEA3 subfamily regulated genes in human mammary epithelial tumor cells; such genes may encode diagnostic and prognostic markers, and molecular targets for drug discovery. To this end we constructed and characterized recombinant human adenoviruses encoding PEA3 or DNPEA3EN with the intent of using such viruses to identify genes that are differentially expressed after infection of normal and tumor derived human breast cell lines.

BODY

PEA3 subfamily transcripts are generally much higher in human breast tumor cell lines compared to normal human mammary epithelial cell strains or immortalized cell lines. Hence we reasoned that we ought to be able to discover PEA3 regulated genes in human breast tumor cell lines by identifying those genes whose expression is repressed by DNPEA3EN in these cells. Correspondingly we reasoned that we might be able to identify PEA3 regulated genes in normal human mammary epithelial cells by identifying genes whose expression is activated by PEA3 in these cells. To perform these experiments we envisioned using non-replicating human adenoviruses to express either PEA3 or DNPEA3EN in the various human mammary epithelial cell types following infection with recombinant adenoviruses encoding one or the other protein. Thereafter we proposed using hybridization of cDNA or cRNA prepared from infected cell RNA to DNA microarrays and Affymetrix GeneChips as a robust means of identifying PEA3 regulated genes.

Our Statement of Work suggested that in year 1 (May, 2002 until June, 2003) we would:
1- Identify candidate PEA3-regulated genes, which are upregulated in normal human mammary epithelial cell lines (MCF10A and hrtr 184) and cell strains (primary human

mammary epithelial cells) after infection with a recombinant adenovirus encoding PEA3 using DNA microarrays and GeneChips; and 2- Construct and characterize a recombinant adenovirus bearing a DNPEA3EN cDNA.

To achieve the first objective requires that human adenoviruses efficiently infect and express the genes of interest (PEA3 or dominant-negative PEA3) in the appropriate mammary epithelial cells. Hence we surveyed the capacity of several untransformed human mammary epithelial cell lines and breast tumor derived cell lines to be infected by a recombinant human adenovirus type 5 vector encoding nuclear-localized, beta-galactosidase from *E. coli* under the control of the human cytomegalovirus early promoter. This adenovirus vector is only able to replicate in permissive human cells expressing the E1A and E1B genes of the virus such as 293 cells. The adenovirus vector is also structurally identical to that encoding PEA3 (or dominant-negative PEA3) and employs the same foreign promoter to direct transcription of the cDNA encoding beta-galactosidase.

To our surprise we discovered that all of the normal mammary epithelial cell lines tested (MCF10A, hTERT-HME1 and htrt184) were refractory to infection by the recombinant adenovirus as revealed by their inability to express beta-galactosidase at the cellular level (data not shown). Less than 1% of the normal mammary epithelial cells expressed beta-galactosidase even after infection at multiplicities as high as 100 plaque-forming units (PFU) per cell (equivalent to over 1000 particles per cell). We also failed to detect PEA3 protein by western immunoblotting following infection of these same normal cell lines with the recombinant adenovirus encoding PEA3 (data not shown). By contrast, three of the four breast tumor cell lines (MDA-MB-361, MDA-MB-468 and BT-549) tested efficiently expressed beta-galactosidase following infection with the recombinant adenovirus. Nearly 100% of the cells from the three, breast tumor cell lines expressed beta-galactosidase after infection at multiplicities of 10 or 100 PFU/cell.

These observations suggested that the identification of PEA3 regulated genes in normal mammary epithelial cell lines might prove problematic. In consequence we accelerated our efforts to derive a recombinant adenovirus encoding dominant-negative PEA3, which could be used to identify genes whose expression is repressed by this protein in human breast tumor cell lines, which express high levels of endogenous PEA3. We are also acquiring additional normal human mammary epithelial cell lines and breast tumor derived cell lines that express no or low levels of PEA3 subfamily proteins to identify PEA3 target genes in these cells following infection with Ad-PEA3.

Our initial attempts to isolate a recombinant adenovirus encoding PEA3 were unsuccessful likely because overexpression of PEA3 interferes with virus replication in permissive human 293 cells perhaps by squelching transcription. Hence we employed a STOP sequence flanked by loxP sites, which contains translation termination codons in all three reading frames, inserted between the CMV promoter and the cDNA encoding PEA3 to derive a stable precursor virus (Ad-STOP-PEA3). Ad-STOP-PEA3 replicates efficiently and to high titer in 293 cells but does not express the PEA3 protein. A recombinant Ad-PEA3 virus was derived from the Ad-STOP-PEA3 virus preparation by

infection of 293 cells that stably express Cre recombinase (293-Cre). Cre recombinase excises the STOP sequences between the loxP sites. Such viral DNA molecules replicate and are ultimately packaged into virions yielding Ad-PEA3. In our experience Ad-PEA3 comprises greater than 95% of the virus produced by the 293-Cre cells; infection of 293 cells with Ad-PEA3 results in high-level expression of PEA3 in these cells (data not shown).

We used the same strategy to isolate Ad-STOP-DNPEA3 and Ad-DNPEA3 (Fig.1). Infection of 293 cells with Ad-DNPEA3 yielded increasing amounts of DNPEA3EN protein in 293 cells with increasing time after infection (Fig. 2). The levels of DNPEA3EN expressed were comparable to those of PEA3 expressed after infection of 293 cells with Ad-PEA3 at the same multiplicity of infection and time course (data not shown). We also attempted to obtain virus stocks of Ad-PEA3 by propagation in 293 cells; we were unsuccessful because overexpressed DNPEA3EN is undoubtedly toxic to virus replication or cell viability. Hence Ad-DNPEA3 is propagated by passage of Ad-STOP-DNPEA3 in 293-Cre cells.

To determine whether the DNPEA3EN protein expressed following infection with Ad-DNPEA3 was biologically active, we transfected 293 cells with a PEA3-responsive reporter plasmid, which bears 5 PEA3 binding sites in head-to-tail array upstream of a TATA box juxtaposed to a cDNA encoding luciferase. Thirty-six hours later the cells were infected with Ad-STOP-DNPEA3 or with Ad-DNPEA3 at varying multiplicities of infection and luciferase activity assayed 12 hours post-infection. The PEA3 responsive reporter (pGL3-5x44-Luc) was expressed in mock-infected 293 cells illustrating that these cells contain PEA3 subfamily or other Ets proteins capable of activating transcription of the luciferase cDNA (Fig. 3). ERM may mediate transcription of the PEA3 responsive reporter in 293 cells because we have detected its transcripts in these cells (data not shown). Surprisingly infection of 293 cells with increasing multiplicities of the Ad-STOP-DNPEA3 recombinant virus led to a dose dependent increase in luciferase expression from the reporter plasmid. These findings suggest that infection of 293 cells with the recombinant adenovirus increases the activity of transcription factors that act on the artificial promoter. Alternatively adenovirus infection may stabilize luciferase mRNA or protein expressed from the reporter plasmid in 293 cells. By contrast infection of the transfected 293 cells with Ad-DNPEA3 led to a multiplicity-dependent reduction in luciferase expression (compare Ad-STOP-PEA3 and Ad-DNPEA3EN at the various multiplicities of infection). At the highest multiplicity of infection used (100 PFU/cell), DNPEA3 reduced luciferase expression 5 fold. Whatever the mechanism whereby adenovirus infection activates expression of the reporter plasmid, these experiments illustrate that DNPEA3 was able to reduce these levels in a dose-dependent fashion implying that it is functional and capable of repressing transcription.

We also infected each of the three human breast tumor cell lines with Ad-STOP-DNPEA3 or Ad-DNPEA3 at different multiplicities of infection, and assessed expression of DNPEA3EN in the infected cell lines by western immunoblotting with antibodies to PEA3. DNPEA3EN was expressed in a dose dependent fashion in each of the breast tumor cell lines infected with Ad-DNPEA3 contingent on the multiplicity of infection

(Figs. 4, 5 and 6). As anticipated DNPEA3EN was not expressed following infection of these same tumor cell lines with the Ad-STOP-DNPEA3 recombinant virus. To ensure that DNPEA3EN is active in breast tumor cell lines, we assayed its capacity to affect expression of luciferase from the PEA3-responsive reporter plasmid in the MDA-MB-468 cell line. In accordance with our earlier findings in 293 cells, infection of the MDA-MB-468 cells with the Ad-STOP-DNPEA3 virus resulted in a multiplicity dependent increase in luciferase expression (Fig. 7). Similarly infection with increasing amounts of Ad-DNPEA3 repressed this adenovirus-dependent increase in luciferase expression in a multiplicity dependent fashion. We also assessed the capacity of PEA3 encoded by Ad-PEA3 to affect luciferase expression in this same experiment. PEA3 was not able to increase luciferase expression above those levels achieved by infection with equivalent multiplicities of Ad-STOP-DNPEA3. It is possible that the levels of endogenous PEA3 subfamily activity or that of other Ets proteins capable of transactivating expression of the reporter plasmid are saturating in the MDA-MB-468 cells; hence increased expression of PEA3 in these cells may have no consequence on reporter gene expression under such circumstances. These data illustrate that DNPEA3EN actively repressed expression of luciferase from the PEA3-responsive promoter in the MDA-MB-468 breast tumor cells and suggested that these breast tumor cells are appropriate to identify genes whose expression is regulated by DNPEA3EN.

To identify PEA3 regulated genes in the three human breast tumor cell lines we infected each cell line with Ad-STOP-DNPEA3 or with Ad-DNPEA3 at a multiplicity of infection of 100 PFU/cell and isolated cytoplasmic RNA 12 hours post infection. Three separate biological experiments were performed for each cell line and RNA harvested for gene expression profiling. At the time of this report only the MDA-MD468 infected cell samples have been analyzed. cRNA was prepared from infected MDA-MB-468 cells and hybridized to Affymetrix HG-133A human GeneChips bearing oligonucleotides representing 22,283 human probe sets (approximately 20% of the probe sets are redundant); hence, the GeneChips contain approximately 17,800 human genes. The hybridization data was processed using Microarray Suite 5.0 and analyzed using MicroDB 3.0 and Data Mining Tool 3.0 from Affymetrix. Raw intensity values from each of the control and experimental arrays were normalized to a target intensity of 150 using Microarray Suite 5.0. The average normalized intensity values for each probe set across three experimental arrays and three control arrays were calculated and the ratio of these averages used to report the linear fold change. For differential expression of each probe set to be deemed statistically significant, two independent statistical analyses were applied in sequence. Pair-wise comparisons were carried out between each experimental and control array for each probe set. The software reports each probe set to be differentially expressed or not based on the behavior of individual oligonucleotides in a given probe set. For those probe sets that passed the pair-wise comparison, a *t*-test was performed in Data Mining Tool 3.0 to provide a confidence interval for each probe set based on consistency among the three biological experiments. Probe sets whose differential expression displayed a confidence interval less than 95% were excluded from the analysis.

Approximately 30% of the probe sets (6,685) were expressed in the infected MDA-MB-468 cells; ~ 15% of these expressed probe sets (1052) were statistically significantly

differentially expressed between MDA-MB-468 cells infected with Ad-DNPEA3 and those infected with Ad-STOP-DNPEA3. The expression of 647 probe sets were reduced and that of 405 probe sets were increased in the MDA-MB-468 cells infected with Ad-DNPEA3 compared to the same cells infected with Ad-STOP-DNPEA3 (data not shown). The expression of 211 probe sets were reduced by a factor of 2-fold or greater, and that of 111 was increased by the same ratio after expression of DNPEA3EN in MDA-MB-468 cells.

We have previously identified candidate PEA3 regulated genes in other cell types including two human cell lines (293 human embryonic kidney cells and SW620 human colorectal tumor cells). Forty of the candidate PEA3 regulated genes identified in the MDA-MD-486 breast tumor cell line were also differentially expressed following infection of SW620 colon carcinoma cells with the Ad-DNPEA3 vector. Twenty-one of the candidate PEA3 regulated genes identified in the MDA-MB-468 cells were also identified as being differentially expressed following the induced expression of an integrated DNPEA3EN cDNA in human 293 cells. However, there were no common genes that were differentially transcribed in response to DNPEA3EN expression in all three human cell lines. It will be interesting to learn whether the same or distinct PEA3 regulated genes are expressed in the three human breast tumor cell lines. We are currently verifying the differential expression of select candidate PEA3 regulated genes identified in the MDA-MB-468 cells; we will complete the analyses of the genes differentially expressed following the expression of DNPEA3EN in the MDA-MB-361 and BT549 breast tumor cell lines shortly. We anticipate that there we will identify PEA3-regulated genes that are commonly differentially expressed in the 3 human breast tumor cell lines and that the products of these genes will include new diagnostic and prognostic markers as well as molecular targets for anti-cancer drug development.

KEY RESEARCH ACCOMPLISHMENTS

1. Identified cell lines derived from human breast tumors that are susceptible to infection by human adenovirus vectors.
2. Constructed and characterized a recombinant adenovirus that encodes a functional, dominant-negative PEA3 protein.
3. Identified candidate PEA3 regulated genes in one human breast tumor cell line (MDA-MB-468).

REPORTABLE OUTCOMES

1. Gene expression profile database of human breast tumor cell line MDA-MB-468.
2. Database of candidate PEA3 regulated genes.
3. Applied for funding to identify genes differentially expressed between metastatic mammary tumors arising in wild type mice and non-metastatic mammary tumors arising in PEA3-null mice based on our experience on this research program.

CONCLUSIONS

During this first year of funding we have recruited a graduate student and technician to the project and made substantial research progress. Indeed we anticipate submitting a manuscript for publication later this year. First we have identified human breast tumor cell lines that support infection by human recombinant adenoviruses and that express cDNAs borne by such virus vectors. Second we previously isolated a recombinant adenovirus capable of expressing PEA3 in human breast tumor cells and have now constructed and isolated another capable of expressing functional dominant-negative PEA3 in these cells. Third, we have identified candidate PEA3 regulated genes in one human breast tumor cell line and are awaiting gene expression profiling data from two additional human breast tumor cell lines. Several of the candidate PEA3 regulated genes identified thus far have previously been implicated in human breast tumors. We imagine that the identification of genes commonly regulated by PEA3 in many different human breast tumor derived cell lines will include those that invariably play key roles in breast cancer. We plan to investigate this potential in succeeding years.

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FIGURES

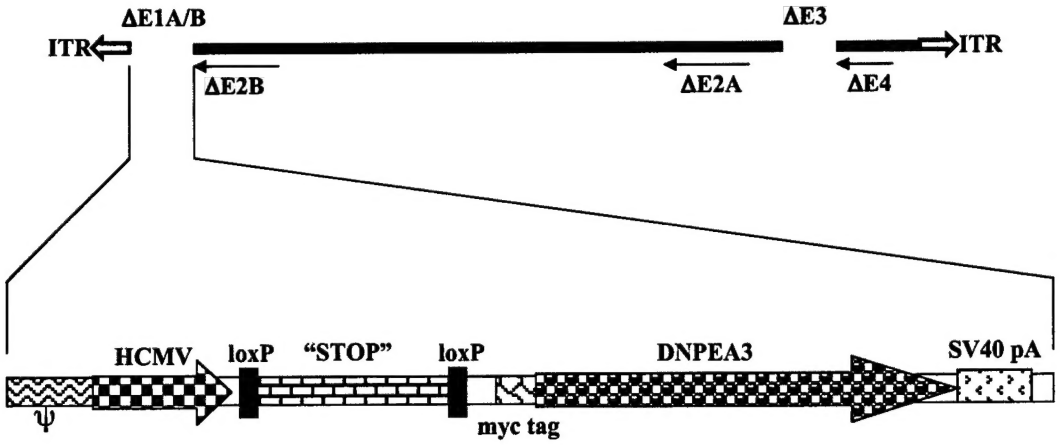

Figure 1. Schematic representation of the adenoviral vector used to express DNPEA3EN. The expression of the recombinant protein is effected by excision of the STOP sequence by passage of the virus through a Cre-recombinase expressing 293 cell line.

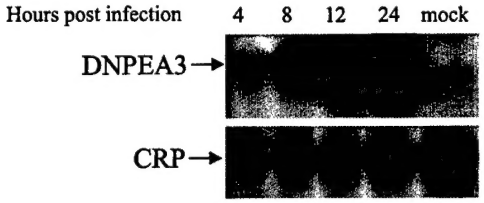

Figure 2. Immunoblot showing expression of DNPEA3EN in 293 cells over a time course post Ad-DNPEA3 infection. Cells were infected at a multiplicity of 10 and whole cell lysates were prepared at selected time points followed by denaturing polyarcylamide gel electrophoresis and immunoblotting. An antibody to the carboxyl-terminus of PEA3 was used to detect DNPEA3. A cross-reacting protein (CRP) serves as a loading control.

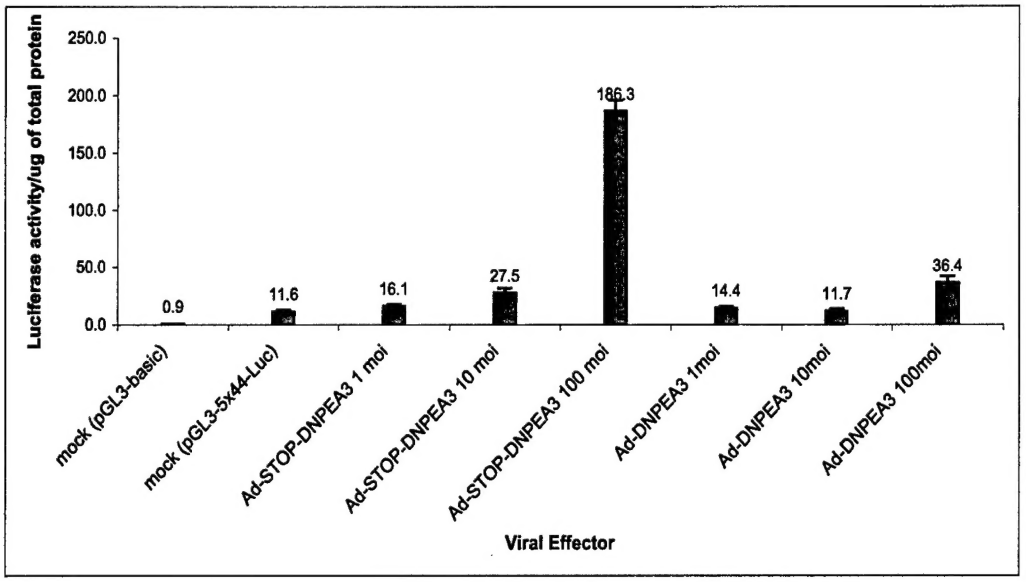

Figure 3. Reporter analysis showing functional activity of DNPEA3EN on the 5x44-Luc reporter in 293 cells. Infection with Ad-STOP-DNPEA3 caused an increase in luciferase activity that was reduced in cells infected with Ad-DNPEA3.

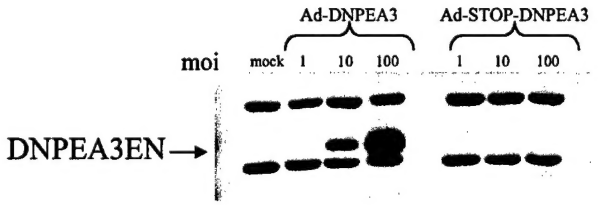

Figure 4. Immunoblot analysis of whole cell lysates from MDA-MB-468 cells 12 hours post infection with Ad-DNPEA3 or Ad-STOP-DNPEA3. Lysates from cells infected at varying multiplicities, from 1-100, were used. DNPEA3EN protein expression was readily detected after infection at a multiplicity of 10 or 100.

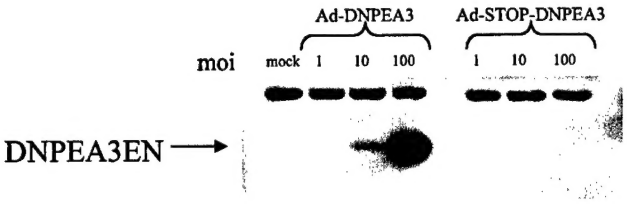

Figure 5. Immunoblot of whole cell lysates from BT-549 cells 12 hours post infection with Ad-DNPEA3 or Ad-STOP-DNPEA3. Lysates from cells infected at varying multiplicities, from 1-100, were used. DNPEA3EN protein was detected after infection at a multiplicity of 10 or 100.

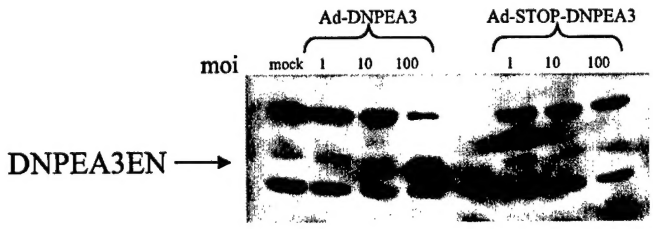

Figure 6. Immunoblot of whole cell lysates from MDA-MB-361 cells 12 hours post infection with Ad-DNPEA3 or Ad-STOP-DNPEA3. Lysates from cells infected at varying multiplicities, from 1-100, were used. DNPEA3EN protein was detected after infection at a multiplicity of 10 or 100.

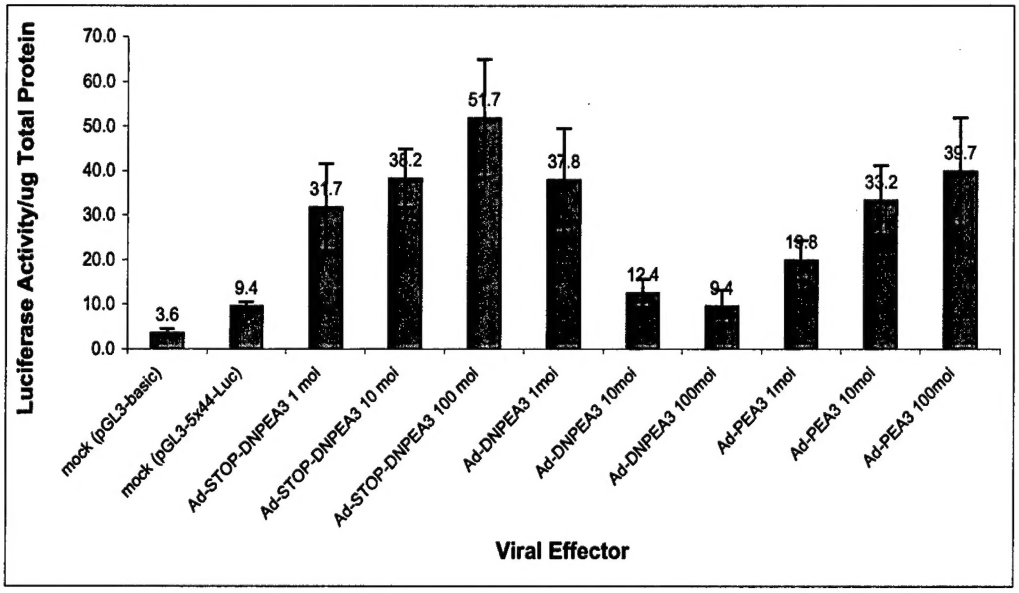

Figure 7. Reporter analysis showing functional activity of DNPEA3EN on the 5x44-Luc reporter in MDA-MB-468 cells. Infection with Ad-STOP-DNPEA3 caused an increase in luciferase activity that was reduced in cells infected with Ad-DNPEA3.